CALIFORNIA
BREAST CANCER RESEARCH PROGRAM
Annual Progress Report
SUMMARY OF RESEARCH PROGRESS

Award # 4FB-0108 Project year (circle): 1st 2nd 3rd Final

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1. Number and list each of the Specific Aims.
2. Indicate any changes in the Specific Aims of your project.
3. Describe in detail the research progress on each of the Specific Aims. Show experimental results, as appropriate.

The report period is either (i) of the pervious year if submitting an Annual Report of (ii) for the entire project period is submitting a Final Report.)

AIM 1 : Identification of partner proteins that interact with various mutant p53 proteins in breast cancer cell lines.

Breast cancer clearly selects for p53 missense mutations, which results in faulty proteins that lack the ability to bind DNA and persist in the cancer cells. Recent evidence suggests that several p53 mutant proteins that most frequently occur in breast cancer have a gain-of-function activity. The hypothesis of this proposal is that the oncogenic mutant p53 proteins that have activation function but lack DNA binding function can induce novel genes and malignant phenotypes by interacting with other cellular proteins which supply DNA binding functions.

For initial two-hybrid screening I used two human p53 mutants (Val143Arg and Arg273His). These mutants were reported to have both dominant negative activities and gain-of-function activities. After I confirmed dominant negative activity of p53 mutants by lifespan extension assay of human fibroblasts, I used full length of those mutants for the bait. Because p53 has a strong transactivation activity, I could not use full length of p53 gene for fusing to Gal4-DB domain. Therefore I fused to Gal4-AT domain. To eliminate false positive, I used a red color screening system which I developed and described in the previous annual progress report. I used HTB-132 (MDA-MB-468) breast cancer cells for constructing library. After several trial of screening I could not get promising candidates except p53 itself which has tetramerization domain in C-terminal and binds each other. As this screening method could not allow me to screen mass population, I switched another two-hybrid system called CytoTrap system from STRATAGENE. CytoTrap two-hybrid system was developed to increase the opportunity for finding unique protein-protein interactions by searching for these interactions in the cytoplasm. In the CytoTrap system, I can use full length p53 for the bait. When I tried to make library for CytoTrap system, I encountered the problem of low ligation efficiency. At that time, Dr. Ladant kindly provided plasmids for bacterial two-hybrid system, then I switched to this system. This system is based on the reconstitution, in an Escherichia coli cya strain, of a signal transduction pathway that takes advantage of the positive control exerted by cAMP. Details of this method were described in previous progress reports. This system also allowed me to use full length of p53 gene for the bait because a signal transduction pathway of cAMP is used to detect interaction. A bacterial two-hybrid system has several advantages compared to yeast system for easy transformation and purification of plasmids. As original paper did not show screening, I started from setting up this system.

Their positive control using leucine zipper motif was also successful in my hand. All colonies became red after 2 days. Then I fused the two human p53 mutants (Val143Arg and Arg273His) and wild type into the T18 protein. I also fused wild type p53 into T25 protein to confirm p53-p53 interaction in this system. Although wild type p53-p53 interaction took one week and mutant

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p53 interaction took 10 days to develop the color of colonies, there was significant difference compared to colonies transformed by empty vectors. There was revertant problem for this system but I successfully suppressed revertant using fosfomycin and streptomycin when I made competent cells. MDA-MB-468 breast cancer cells were used for making cDNA library and cDNAs were fused to T25. Then, I performed several screenings but I could not get positive candidates, plasmids from which, could retransform. I also still picked up positive colonies that were found out to be revertant that did not contain library plasmids.

While I was trying several methods to use full length of p53 for the bait, I also performed GAL4 based conventional yeast two-hybrid screening using truncated p53 that lacks activation domain at the same time. Although I could not get promising candidate from truncated mutant p53, I could successfully cloned two candidates from truncated wild type p53. Plasmids derived from these candidates could retransform. One is Human Proteasomal Subunit HsC8 and the other is metal response element-binding transcription factor-1(MTF-1). Then I tried immunoprecipitation to confirm interactions but interaction was negative. I used commercial antibody for detecting HsC8. I made HA tagged MTF-1 for detecting MTF-1. Although I did not perform other methods like GST pull down, I concluded these candidates were false positives.

AIM 2 : Characterization of p53-interacting proteins

Because I could not get candidates, I did not proceeded to AIM2.

Alternative AIM : Role of a potential human tumor suppressor, DMP1, and its new spliced variant in human breast cancer

When the initial project looked unsuccessful, I started another related project to study the role of p53 in breast cancer. Recent research has demonstrated that a p53 pathway is critical in preventing immortalization of normal epithelial cells. It has become clear in the last several years that this pathway is perturbed in majority of breast cancers. Recently p14/ARF was found to act as an upstream protein for p53. p14/ARF prevents degradation of p53 and therefore can promote growth arrest, senescence or apoptosis in response to unregulated cell proliferation. Deletion of p14/ARF is frequently observed in many types of breast cancer cell lines such as MDA-MB231. Mice with germ line inactivation of p19/ARF, which is the mouse homologue of human p14/ARF, are highly tumor prone. Despite this evidence that p14/ARF plays an important role in preventing immortalization, little is known about what induces p14/ARF.

Recently, however, a protein that directly induces p14/ARF, DMP1, was identified. DMP1 is a transcription factor and mouse DMP1 directly binds the p19/ARF promoter and causes cell cycle arrest in normal mouse embryo fibroblasts (MEFs) but not MEFs from p19/ARF knockout mice. Furthermore, DMP1 null-mice were made, and neonatal treatment with carcinogens, or ionizing radiation induced tumors that were not observed in similarly treated control mice and MEFs from DMP1 null-mice did not senesce in culture.

I started to study DMP1 because elucidating the link between p53, p14/ARF, and DMP1 in p53 pathway has potentially great significance in understanding the mechanism of tumor suppression of normal breast epithelial cells and breast cancer development,.

Based on this sequence recently reported in Genbank, I independently cloned human DMP1 from testis using PCR. When I analyzed expression of DMP1 mRNA by RT-PCR in breast cancer cell line(MCF7), however, I found an additional band, suggesting the existence of a spliced variant. From analysis of the genomic sequence of human chromosome 7q21.1-q21.2, recently submitted to GenBank, I determined that the normal form of human DMP1 gene contains 18 exons. The splice variant, in contrast, lacks exon 17. This results in a reading frame

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shift and a different and shorter C-terminal portion from the other form of DMP1. To confirm that a splice variant did not arise from a PCR artifact, I performed RT-PCR using several different sets of primers. 11 PCR products, including full length, showed the existence of the same splice variant determined by size. Two products were sequenced completely and both lacked exon 17. I also found a spliced variant that has exactly the same sequence in the EST database. This EST clone (GenBank accession number BE268190) was not generated by PCR and was derived from a Burkitt lymphoma cell line.

A strong transcription factor activation domain was reported to be located in the C-terminus of mouse DMP1, and truncated C-terminal mouse DMP1 completely lacks transactivation activity but retains DNA binding activity. This suggests that the spliced variant of human DMP1, with its altered C-terminal domain, may lack transactivation activity and function as dominant negative fashion.

I next examined the expression of both the long and short forms of DMP1 in normal and immortalized breast epithelial cells, using semi-quantitative RT-PCR. Although all immortalized and transformed breast epithelial cells (MCF10A, T47D, MCF7) had significant levels of the spliced variant, normal epithelial cells (184 cell line) did not express the spliced variant at detectable levels after 26 cycles. In contrast, all cell lines examined, including normal breast epithelial cells, expressed longer form of DMP1. These data suggested that expression of the spliced variant is closely correlated with immortalization.

I hypothesize that p14/ARF induction by DMP1 is essential for both normal replicative senescence and growth arrest induced by oncogenic stimulation of normal breast epithelial cells, a compensatory mechanism that prevents immortalization. I also hypothesize that while this role is played by longer form of DMP1, which thus acts as a tumor suppressor, an alternatively spliced form acts as an oncogene to inhibit functions of longer form. Thus when the spliced form is highly expressed, longer form may lose its ability to induce p14/ARF, with the result that normal breast epithelial cells bypass the senescence checkpoint, and become susceptible to immortalization. If my hypothesis is correct, the spliced variant may also be a useful marker of an early step in the development of breast cancer.

I will determine the effect of introduction of each of these genes to change the ratio of these protein in the cell on the fate of normal or immortalized cells; and quantify the levels of both forms in normal and immortalized breast epithelial cells, in normal epithelial cells undergoing senescence, and in normal and cancerous human breast tissue biopsies.

Summary of Research Progress (continued)